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Inhibition of NF-κB specific transcriptional activation by PNA strand invasion

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ABSTRACT

Peptide nucleic acid (PNA) strand invasion offers an attractive alternative to DNA oligonucleotide directed triplex formation as a potential tool for gene inhibition. Peptide nucleic acid has been shown to interact with duplex DNA in a process which involves strand invasion of the duplex and binding of one of the DNA strands with two PNA oligomers. By blocking the interaction of a transcription factor with 5′ regulatory sequences, PNA might specifically down-regulate gene activity. Here we demonstrate that PNA is capable of specifically blocking interaction of the transcription factor NF-κB with the IL2-Rα NFκ-B binding site *in vitro*. We further demonstrate that this interaction is sufficient to prevent transcriptional transactivation both *in vitro* and when transfected into cells in culture.

INTRODUCTION

Peptide nucleic acid (PNA) oligomers form highly stable complexes with complementary DNA (1–3). Duplex DNA is also stably bound by PNA in a process which involves strand invasion. Presumably this occurs when two homopyrimidine PNAs bind the complementary homopurine DNA strand, displacing the other DNA strand (4,5). The stability of the PNA/DNA/PNA triplex is sufficient to inhibit restriction endonuclease cleavage of duplex DNA *in vitro* (6). In addition PNA effectively inhibits both prokaryotic and eukaryotic polymerases *in vitro* (7,8). Eukaryotic transcription factor binding domains offer another potential target for strand invasion.

NF-κB was originally described as a factor that bound to a short enhancer sequence important for the activity of the κ light chain immunoglobulin gene (9). Because it was constitutively present only in those B cells of the appropriate stage for light chain expression, NF-κB appeared to be a tissue restricted transcription factor. It is now clear however that NF-κB plays a more general role in transcription (reviewed in ref 10). It has been determined that NF-κB is a heterodimer consisting of two distinct subunits, p50 and p65 (9,11). Cloning of these subunits has revealed that they belong to the Rel family of DNA binding proteins which includes c-rel, v-rel, Rel B, p65, p50 and p49 (12).

Although active NF-κB is expressed in the nucleus of mature B cells, it has been found to be present in non-B cells in a covert cytoplasmic form which can be induced to translocate to the nucleus by phorbol esters (13). In stimulated T-lymphocytes, activation of NF-κB results in the induction of the interleukin-2α receptor (IL-2Rα) (14). In addition, NF-κB has been shown to regulate the expression of β-IFN, MHC-class II, IL-6, HLA, TNF-α, lymphotoxin and several viral promoters including the HIV-1 enhancer which has two NF-κB binding sites (15). Each of these genes contains a highly conserved NF-κB binding site with the consensus sequence RGGGRMTYYCC.

The 3′ end of the NF-κB site of the IL-2Rα promoter is overlapped by a 15 base homopyrimidine stretch not present in other known NF-κB regulated genes. Targeting of this site with triplexing oligonucleotides has previously been reported by Grigoriev *et al.* who were able to demonstrate inhibition of NF-κB binding and transcriptional activation of the IL-2Rα promoter using an acridine-derivatized, methylcytosine substituted DNA oligonucleotide (16). Strand invasion by PNA offers an attractive alternative to third strand binding by DNA. In the present study we demonstrate specific inhibition of NF-κB binding and transactivation by a 15 base homopyrimidine PNA targeted to the IL-2Rα promoter. The affinity of the PNA for the target was sufficient to allow for specific strand invasion under physiological conditions. In addition, invasion of the duplex by PNA prevented NF-κB binding *in vitro* and inhibited transactivation of the IL-2Rα promoter in eukaryotic nuclear lysates. Finally we have demonstrated that NF-κB dependent transactivation of an IL-2Rα reporter gene construct expressed in cultured cells was specifically inhibited when PNA was bound to the transfected plasmid.

MATERIALS AND METHODS

PNA and oligonucleotide synthesis

Peptide nucleic acids were synthesized and purified as previously described (1,17,18) and were analyzed by HPLC and mass spectrometry. DNA oligonucleotides were synthesized using an

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Applied BioSystems 380B automated synthesizer and standard phosphoramidite chemistry.

Synthesis of duplex DNA targets

DNA target A, 5′- GAT GAG TTC GTG TCC GTA CAA CTG GGG AAT CTC CCT CTC CTT TTA GGC GCT GTG GCT GAT TTC GAT AAC C-3′, consists of the IL-2Rα NF-κB site and homopyrimidine sequences 3′ to the NF-κB site which make up the PNA strand invasion site (–243 to –265) flanked by universal primer sequences (NF-κB site and homopyrimidine stretch underlined). Target B, 5′- GAT GAG TTC GTG TCC GTA CAA CTG GTC TCC CTC TCC TTT TGG CGC TGT GGC TGA TTT CGA TAA CC-3′, contains only the strand invasion site; the NF-κB site has been deleted. Duplex DNA targets for *in vitro* determination of PNA affinity and NF-κB binding were produced by PCR amplification of each oligonucleotide using ^{32}P end labeled universal primers. After 30 rounds of PCR amplification, the full length duplex targets were purified by native polyacrylamide gel electrophoresis, then resuspended in assay buffer.

PNA strand invasion and binding

Binding of PNA and NF-κB to duplex DNA targets was measured by gel mobility shift assay (19). For PNA binding, radiolabeled DNA target (~10 pM) was incubated with increasing concentrations of PNA in either TMTB (100 mM Na^+, 10 mM phosphate, 0.1 mM EDTA) or TE (10 mM Tris, 0.1 mM EDTA, pH 7). Following the incubation, PNA bound duplex was separated from free target by electrophoresis through an 8% native polyacrylamide gel. We define EC_{50} as the PNA concentration at which half of the target is bound. In TMTB, association rates were so slow we were unable to demonstrate thermodynamic equilibrium. Thus, reported values of EC_{50} depend upon the time of hybridization, which is specified for each experiment.

To examine inhibition of NF-κB binding, PNA was first allowed to bind the DNA target in TE as described above, then the buffer conditions were adjusted to allow binding of the p50 homodimer (TSB buffer: 10 mM HEPES pH 7.9, 50 mM KCl, 0.2 mM EDTA, 2.5 mM DTT, 10% glycerol and 0.05% NP-40) and 200 ng of protein (recombinant purified p50 subunit, Promega Biotech) were added. Following a 30 min incubation at 37°C, NF-κB and PNA bound DNA duplex were separated from free by electrophoresis as described above.

Plasmid construction

Two oligonucleotides with the sequences 5′-CAG GGG AAT CTC CCT CTC CTT TTC AGG GGA ATC TCC CTC TCC TTT TC-3′ and 5′-TCG AGA AAA GGA GAG GGA GAT TCC CCT GAA AAG GAG AGG GAG ATT CCC CTG GTA C-3′ were annealed in PBS. The resulting duplex contains tandem repeats of the IL-2Rα NF-κB site and homopyrimidine stretch (–243 to –265) and *Kpn*I and *Xho*I sticky ends. It was ligated into the vector pGL2-Promoter (Promega) at the same restriction sites, placing the IL-2Rα NF-κB site just upstream of the SV40 promoter driving the expression of luciferase. SV40 enhancer sequences are not present in this vector.

In vitro transcription

For *in vitro* transcription assays PNA was pre-incubated at 37°C with 1 μg of *Eco*RI linearized plasmid overnight in 10 μl of TE. DNA (150 ng) was removed and transcribed in a 25 μl HeLa Scribe (Promega) reaction essentially as outlined in the manufacturer's protocol. All transcription reactions were supplemented with 1 mM DTT, 0.5 μl 3000 Ci/mmol-[α-^{32}P]GTP and poly dI.dC (0.2 mg/ml) as a non-specific competitor. As a source of NF-κB, nuclear extracts prepared from uninduced or PMA induced Jurkat cells (Santa Cruz Biotech) were used in the place of the 1× buffer supplied in the HeLa Scribe kit. The reactions were incubated 1 h at 37°C then phenol extracted, followed by ethanol precipitation of the RNA. The RNA pellet was resuspended in 10 μl of RNA gel loading buffer and electrophoresed through a 5% denaturing acrylamide gel. Following electrophoresis the gel was dried and exposed to film at –80°C or analyzed using a Molecular Dynamics PhosphorImager.

Cell culture

HeLa cells were maintained in DMEM with 10% FCS. For activity in cell culture 3 μg of plasmid was incubated overnight with PNA at the concentrations specified in 30 μl TE. The following morning 10 μl of the plasmid/PNA was digested for 30 min with 5 U each of *Eco*RI and *Xho*I. Restriction protection of the *Xho*I site by the invaded PNA was visualized by electrophoresis through a 1% agarose gel and ethidium bromide staining. The remaining 20 μl of the plasmid/PNA was brought to a volume of 100 μl with OptiMEM media. This was combined with 100 μl of OptiMEM containing 10 μg of LipofectAmine reagent (Gibco-BRL) and incubated for 20 min at room temperature. This mixture was then divided between duplicate wells of a 6-well plate containing 50% confluent HeLa cells in 800 μl of OptiMEM. Following a 6 h incubation at 37°C the media was removed and replaced with fresh DMEM containing 10% FCS. Immediately following the transfection, PMA (50 ng/ml) and PHA (0.5 μg/ml) were added to the cells to induce production of NF-κB. The cells were incubated overnight at 37°C. The following day cells were harvested and assayed for luciferase activity as described elsewhere (19).

RESULTS

Two 15 base homopyrimidine PNAs, IP-8129 and IP-9151 (Table 1), were designed to bind the homopyrimidine/homopurine region which overlaps the 3′ end of the IL-2Rα NF-κB site (Fig. 1). Strand invasion of target B, which contains the IL-2Rα homopurine stretch but not the NF-κB site, was measured in 100 mM Na^+ buffer (TMTB) by gel shift. Invasion rates were determined to be slow in TMTB. At 10 μM PNA, binding of IP-9151 to target B did not begin to plateau until 5 days at 37°C (data not shown). For IP-9151 the EC_{50} after a 2 day incubation at 37°C was determined to be 3.8 μM, while the antiparallel PNA, IP-8129, showed little affinity for the same target even at concentrations up to 40 μM. S1 nuclease mapping of the PNA bound target revealed that the homopyrimidine DNA opposite the homopurine PNA binding site was displaced and the DNA flanking the PNA binding site remained duplex (data not shown).

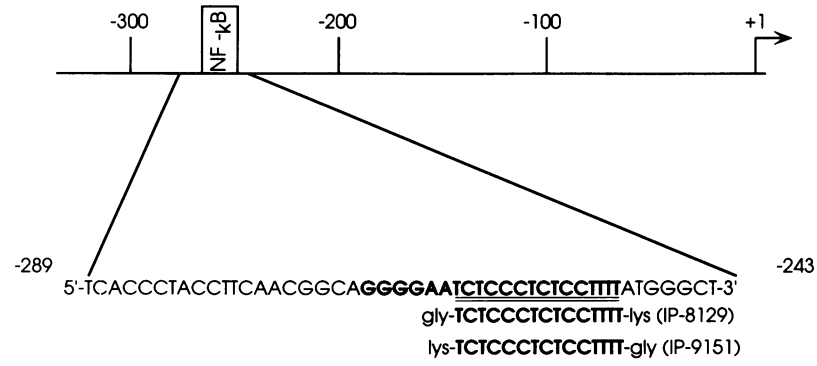

Figure 1. IL2-Rα 5′ UTR. The sequence of the region surrounding the NF-κB site and homopyrimidine stretch is shown with the NF-κB site in bold and the PNA target site underlined. The binding orientations of PNAs IP-9151 and IP-8129 are shown beneath the strand invasion sight.

Table 1. PNA oligomers used in study

ISIS no.	Target	Sequence
IP-8129	IL2-Rα, antiparallel to purine strand	gly-TCTCCCTCTCCTTTT-lys
IP-9151	IL2-Rα, parallel to purine strand	gly-TTTTCCTCTCCCTCT-lys
IP-11204	Random homopyrimidine	gly-TCTCTCTCTCTCTCT-lys
IP-8130	Random mixed sequence	gly-TGTACGTCACAACTA-lys

By analogy to peptides, PNA sequences are written from amino to carboxy terminus. The carboxy terminus is a carboxy-amide.

To determine the effect of strand invasion on NF-κB binding *in vitro*, PNAs were pre-incubated with a 70 bp PCR generated DNA duplex, target A, which includes positions –243 to –265 of the IL-2Rα promoter (both κB site and homopurine invasion site). Pre-incubations were performed in TE instead of TMTB due to the slow kinetics of invasion in the higher salt buffer. Following a 2 h pre-incubation the buffer conditions were adjusted to TSB buffer and 200 ng of purified NF-κB p50 was added. The amount of p50 bound in the presence of increasing concentrations of PNA was visualized by gel shift. The results are shown in Figure 2. In the absence of the p50 protein the EC_{50} for duplex strand invasion was ~700 nM (lanes 1–6). Length matched control PNAs, IP-11204 and IP-8130, did not bind the target at the highest concentration tested (10 μM; lanes 7 and 8). Purified p50 specifically bound target A in the absence of PNA, presumably as a homodimer (lane 11). There was virtually no p50 binding to target B, which contains the PNA binding site but does not contain an NF-κB site (lane 19), however this target was completely bound at 10 μM PNA 9151 (lanes 10 and 20). Binding of the p50 to target A was inhibited by 50% (IC_{50}) by a PNA concentration equal to the EC_{50} for strand invasion (lanes 11–16). Again the control PNAs at 10 μM had no effect on p50 binding to the target (lanes 17 and 18). Peptide nucleic acid 9151 also effectively inhibited the binding of recombinant purified p49 and p65 produced by *in vitro* transcription/translation (data not shown). These data demonstrate that PNA strand invasion results in specific disruption of protein binding to the NF-κB site.

We also looked at the effects of disruption of p50 binding in an *in vitro* transcription assay. A 47 bp synthetic DNA duplex consisting of tandem repeats of the IL-2Rα NF-κB region was cloned immediately upstream of the SV40 promoter in the vector pGL2-Promoter, a luciferase reporter vector lacking SV40 enhancer sequences. The resulting plasmid, pGL-κB, was linearized with *Eco*RI then pre-incubated in TE for 2 h with PNA at concentrations of 3.3, 1 or 0 μM. As a control pGL2-C, a plasmid containing both SV40 promoter and enhancer sequences, was treated identically. Following pre-incubation, the plasmids were transcribed in a HeLa cell extract supplemented with extract prepared from PMA induced Jurkat cells as a source of NF-κB. Extracts from Jurkats not treated with PMA were used to control for basal levels of transcription. The transcripts were then visualized by electrophoresis through a denaturing polyacrylamide gel. The results are shown in Figure 3. The amount of full length (870 nucleotides) run-off transcript was increased in the presence of PMA induced Jurkat extract (compare lane 1 with lane 2). PNA IP-9151 inhibited the transactivation completely at 3.3 μM, but had no effect at 1 μM (lanes 3 and 4). The control target, pGL2-C, was also transactivated by the PMA induced Jurkat extract (compare lanes 9 and 10), however, 9151 had no effect on the level of transcriptional transactivation at either the 1 or 3 μM concentrations (lanes 11 and 12). In addition, PNA controls IP-8130 and 11204, showed no inhibition of transactivation of pGL-kB (lanes 5–8).

Finally, we looked at the ability of PNA to block NF-κB mediated transactivation in cells. pGL-κB and pGL2-C were pre-incubated with PNA overnight in TE buffer. An aliquot of the PNA bound plasmid was digested with *Eco*RI and *Xho*I. This releases a fragment of ~800 bp from the unbound plasmid. However, the PNA binding site overlaps the *Xho*I site by 1 bp, preventing cleavage by this enzyme if PNA is bound. Only PNA IP-9151 showed inhibition of enzyme activity and inhibition was observed only against the pGL-κB target which contained the PNA binding site (data not shown). The remainder of the PNA/plasmid complex was transfected into HeLa cells as described in Materials and methods. Following transfection, NF-κB production was induced by the addition of PMA/PHA. The cells were incubated overnight and luciferase activity measured the following day. The results, shown in Figure 4, are presented as the luciferase activity in light units relative to uninduced controls. At a PNA concentration of 3.3 μM, IP-9151 inhibited transactivation of pGL-κB completely while the control

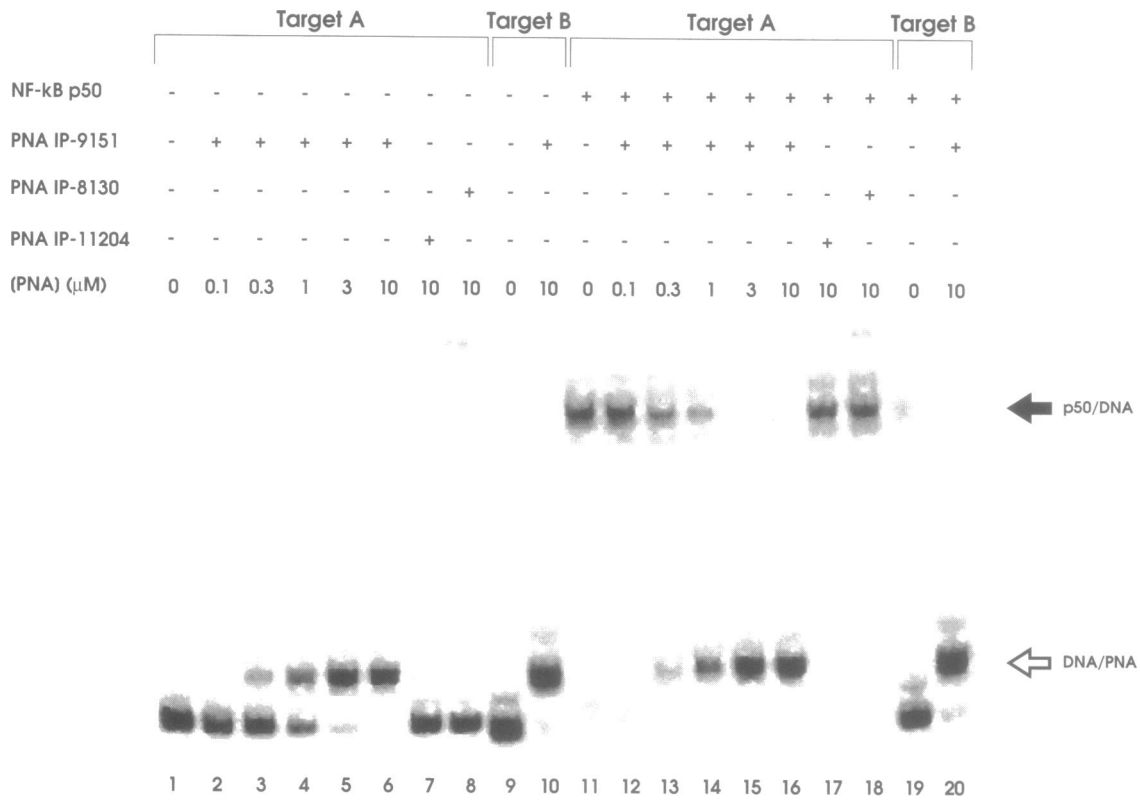

Figure 2. Effect of PNA IP-9151 on NF-κB binding *in vitro*. An end labeled 70 bp PCR generated IL2-Rα fragment, target A, which contains the NF-κB site and PNA target (lanes 1–8 and 11–18) or target B, a fragment without the NF-κB site (lanes 9–10 and 19–20), was pre-incubated in TE buffer with PNA at the indicated concentrations. Following the pre-incubation, buffer conditions were adjusted to allow p50 binding (lanes 11–20). PNA and p50 bound target were separated from free by electrophoresis on a non-denaturing polyacrylamide gel. The open arrow indicates the position of the strand invaded duplex, while the filled arrow indicates the position of the p50 bound duplex.

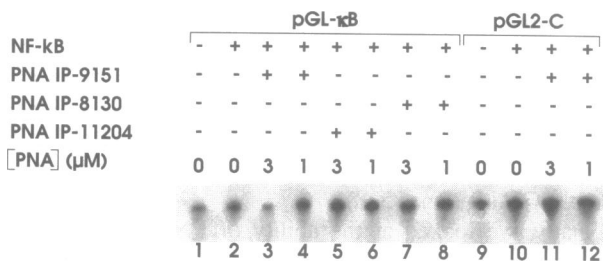

Figure 3. Inhibition of *in vitro* transcriptional activation by PNA IP-9151. The plasmid pGL-κB was linearized with *Eco*RI then pre-incubated in TE buffer with PNA. Following the pre-incubation the plasmid was transcribed in a HeLa cell extract supplemented with PMA induced Jurkat extract as a source of NF-κB (lanes 2–8 and 10–12). The 870 base run off transcript was visualized by electrophoresis on a denaturing polyacrylamide gel.

PNAs had little effect. None of the PNAs had an effect upon the transactivation of the control construct, pGL2-C.

DISCUSSION

The main objective of this work was to determine if PNA strand invasion of transcription factor binding sites results in specific disruption of transcriptional transactivation. Since PNA strand invasion of duplex DNA has only been demonstrated using pyrimidine rich PNAs (5,6,8), a homopurine target was selected which lies in close proximity to a known transcription factor binding site. The NF-κB site of the IL-2Rα promoter overlaps with a 15 base homopurine/homopyrimidine duplex (Fig. 1). *In vitro* binding experiments using a radiolabeled fragment of this region demonstrated that binding and invasion of duplex DNA were sequence specific and that the orientation of the PNA relative to the DNA was important. In a buffer at physiological ionic strength the parallel PNA bound much more tightly than the antiparallel, however the kinetics of invasion were determined to be very slow. Slow invasion at physiological ionic strengths has been reported recently for homopyrimidine PNA (18). PNA bound the duplex with sufficient affinity to specifically prevent binding of the NF-κB transcription factor at a site which overlaps the PNA invasion site. Binding of PNA to duplex target correlated perfectly with inhibition of transcription factor binding.

PNA is able to specifically inhibit transactivation *in vitro* under certain conditions. We were not able to demonstrate specific activity when the PNA was added directly to transcription extracts, which have a KCl concentration of 100 mM at pH 7.9. Presumably, this was due again to the slow kinetics of invasion in physiological salt. Specific activity was observed when PNA was incubated with the DNA target in low salt buffer prior to addition to the nuclear extracts. By pre-incubating the DNA with PNA in low salt, we were able to reduce PNA concentrations to

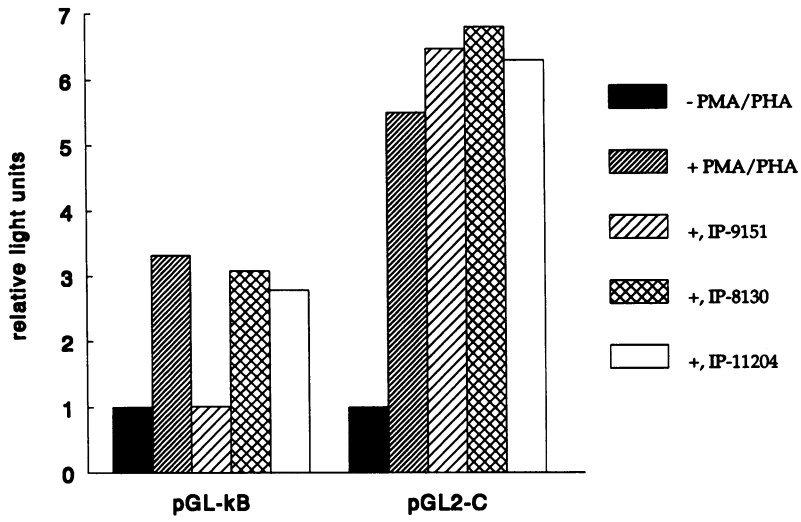

Figure 4. Inhibition of NF-κB induced transactivation of pGL-κB in HeLa cells. Plasmids pGL2-C or pGL-sκB were pre-incubated with PNA at 3.3 μM in TE buffer. The plasmid/PNA complex was then transfected into HeLa cells. NF-κB was induced following the transfection by the addition of phorbol esters (+). Cells were harvested 24 h later and assayed for luciferase activity. The luciferase activity has been normalized to the uninduced control (first bar of each set). These data are representative of triplicate experiments.

a level where there was little non-specific effect on the transcription reaction. It should be noted that transcriptional activation by PMA induced Jurkat extract was not quantitatively reproducible between experiments, probably due to variability in NF-κB levels between different preparations of extract. It was consistently observed, however that the amount of transcription was reduced below uninduced levels by the sequence specific PNA, IP-9151 (compare lanes 1 and 3, Fig. 3). This result may be due to PNA binding inhibition affecting association of other transcription factors thereby reducing the overall levels of transcription. For example, the PNA binding site of the IL-2Rα gene overlaps the 5′ end of the serum response element (SRE) (20).

Finally, we looked at the ability of PNA to prevent NF-κB mediated transactivation in a cell culture model. While direct addition of PNA to previously transfected cells had no specific effects on expression, specific activity was observed when the plasmid was pre-incubated with PNA prior to transfection. It is noteworthy that once bound the PNA/DNA/PNA triplex was sufficiently stable to produce an effect even after 24 h in cell culture. This is consistent with previous observations that off-rates for PNAs complexed with duplex DNA targets are exceedingly slow (18).

The ability of PNA to inhibit transcription *in vitro* suggests its value as a potential antigene therapeutic agent. In using PNA as an antigene agent kinetic barriers must be considered. The relatively high ionic strength and pH of physiological buffers are not optimal for strand invasion (5,6,8,21). The effects of pH are observed for PNAs with high cytosine content (T.A.V. unpublished) and may be alleviated by targeting adenosine rich regions, avoiding dG/dC duplexes. Alternatively, modified residues could facilitate binding *in vivo*. For example, pseudo-iso-cytidine substituted DNA will form triplexes at physiological pH (22,23). The same substitution in PNA has also been evaluated and allows for efficient strand invasion at neutral pH (24).

At neutral pH the more imposing problem is that of the ionic strength of the cellular environment. Specific inhibition of *in vitro* transcription and cell culture models is most efficient when pre-incubations are carried out at ionic strengths less than those typically found in cells. This appears to be due to reduced binding rates at higher ionic strengths. Therefore, it is possible that PNA will strand invade under physiological conditions if given sufficient time to overcome kinetic barriers. If these binding rates are too slow for effective antigene activity *in vivo*, strategies to enhance rates of strand invasion could be adopted. For example, metabolically active regions of the genome could be targeted. Another possibility would be use of bis-PNAs. Griffith *et al.* have reported PNA strand invasion with EC_{50}s as low as 50 nM in 100 mM Na^+ (18). This was accomplished using bis-PNAs; an approach in which the two PNA strands are linked together to reduce entropy and convert binding into a bimolecular process. It is possible that bis-PNA could also be capable of strand invasion at low concentrations under physiological conditions. With improved binding under physiological conditions, PNA presents a unique opportunity for regulation of gene expression by site specific inhibition of transcriptional transactivation.

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